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The MAR1 transporter is an opportunistic entry point for antibiotics

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The vast quantities of antibiotics used in modern agriculture contaminate the environment and threaten human health. Recent studies have shown that crop plants grown in soil fertilized with manure from antibiotic-treated animals can accumulate antibiotic within the plant body, thus making them an additional antibiotic exposure route for consumers. Until recently, mechanisms of antibiotic entry and subcellular partitioning within plant cells were virtually unknown. We have uncovered and characterized a transporter gene in *Arabidopsis thaliana*, *MAR1*, which appears to control antibiotic entry into the chloroplast. Antibiotic resistance via *MAR1* is specific to the aminoglycoside class, and is conferred by loss-of-function mutations, which is rather unusual, since most transporter-based antibiotic resistance is conferred by overexpression or gain-of-function mutations in efflux pumps with poor substrate specificity. Since *MAR1* overexpression lines exhibit various iron starvation phenotypes, we propose that MAR1 transports an iron chelation molecule that is mimicked specifically by aminoglycoside antibiotics, and this facilitates their entry into the chloroplast. Knowledge about MAR1 enhances our understanding of how antibiotics might enter the plant cell, which may aid in the production of crop plants that are incapable of antibiotic accumulation, as well as further the development of new plant-based antibiotic resistance markers.

Key words: antibiotic, contamination, transport, import, chloroplast, membrane, iron, chelation, nicotianamine

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Antibiotic Contamination of Crop Plants: An Emerging Public Health Problem

The amount of antibiotics used non-therapeutically in agriculture is estimated to be eight times greater than the amount used in all of human medicine,[1] and accounts for about 70% of total antibiotic use in the United States.[2] Until recently, major concerns about antibiotic use in agriculture have been related to their contamination of animal-based food products—such as milk and meat—and their pollution of the water supply via farm runoff. However, many antibiotics are not well absorbed in the animal gut and are excreted largely unchanged in manure,[3,4] some retaining 75% of their activity after 2 years in soil.[5] Despite this known presence and persistence, there are no guidelines on the presence of antibiotics in manure,[6] and crop plants fertilized with antibiotic-contaminated manure are now emerging as additional antibiotic exposure routes. There is a growing body of work describing the ability of various plants to accumulate measurable levels of antibiotic after growth on contaminated soils,[7-10] but unanswered questions remain about the molecular nature of this uptake, especially for hydrophilic antibiotics, which do not readily diffuse across membranes.

Antibiotic Resistance in Plants: A Poorly Studied Area

It is widely recognized that plants are sensitive to many antibiotics, and this fact has been exploited for the benefit of both basic and applied plant science to produce transgenic plants. Transgenic plant selection

systems are often based on expression of bacterial genes, which typically provide resistance to antimicrobial compounds via enzymatic inactivation.[11-13] Most of these enzymes are specific for one or a few particular aminoglycoside antibiotics, which generally target the translational machinery of prokaryotes. Because the eukaryotic organellar translational machinery is prokaryotic in nature, these antibiotics target chloroplast and mitochondrial translation in plants. Until now, endogenous, high-level resistance to aminoglycosides in plant lines has typically been found to be due to specific changes in organellar ribosomal subunits.[14,15]

Drug resistance in bacteria has been well studied, and is often conferred by expression of multidrug efflux transporters with low substrate specificity.[16] These transporters encompass several protein families including ATP binding cassette (ABC) transporters, the major facilitator superfamily (MFS), the multidrug and toxic compounds efflux (MATE) family and others.[17] However, in plants, there are only a few reports of antibiotic resistance that are based on overexpression of efflux transporters. In one study, it was shown that overexpression of an endogenous *Arabidopsis thaliana* ABC transporter gene, *AtWBC19*, confers kanamycin resistance in plants.[18] Levels of resistance were similar to levels attained through expression of the bacterial neomycin phosphotransferase II (*nptII*), which is one of the most commonly used selectable markers in plants. The discovery and characterization of *AtWBC19* has sparked the hope that plant-based antibiotic transport proteins may be promising new candidates for selectable markers.[19]

Since *AtWBC19* is likely to be involved in antibiotic sequestration to the vacuole, it can be overexpressed for use as a marker. However, antibiotics must enter the cell in order to function, and a block of entry may also be sufficient to generate resistance. Once plant-endogenous antibiotic import proteins are uncovered and characterized, additional markers may be developed via RNAi-mediated downregulation of these proteins. Perhaps not surprisingly, movement of aminoglycoside antibiotics across the bacterial inner membrane involves energy-dependent transport,[20,21] and recent work suggests that uptake of antibiotic into plants is also an energy-dependent process.[22] Unfortunately, the specific plant transporter proteins capable of recognizing and importing antibiotics have remained unknown until now.

MAR1: A Gateway for Antibiotics into Plant Chloroplasts

We have recently uncovered and characterized a transport protein of *Arabidopsis thaliana*, MAR1, which we have shown is capable of transporting multiple aminoglycoside antibiotics.[23] The original EMS mutant, *mar1-1*, which has a single amino acid change in a putative transmembrane domain of the protein, was resistant to the aminoglycosides kanamycin, tobramycin, gentamicin, streptomycin, amikacin and apramycin. However, *mar1-1* showed no resistance to the non-aminoglycosides spectinomycin, chloramphenicol, lincomycin and tetracycline, or to aminoglycosides that inhibit both prokaryotic and eukaryotic translation (G418, hygromycin and paromomycin). Two independent T-DNA insertions in *MAR1* were able to phenocopy the multiple resistance phenotype of *mar1-1*, while *MAR1* overexpression lines were hypersensitive to aminoglycosides. Thus, *MAR1* stands out as rather unusual in that it appears to recognize only one specific group of antibiotics, and resistance to these antibiotics is conferred by loss-of-function mutations.

Using a MAR1-YFP fusion protein, we went on to show that MAR1 localizes to the chloroplast, and is likely to be an inner membrane protein that allows entry of aminoglycoside antibiotics into the stroma, where they interfere with organellar (prokaryotic) translation.[23] Thus, when *MAR1* is disrupted, resistance is not seen to aminoglycosides that would interfere with cytoplasmic (eukaryotic) translation, as their entry into the cytoplasm is not barred. Evidence for the antibiotic transport functionality of MAR1 was uncovered using both yeast and isolated chloroplasts. Yeast expressing *MAR1* cDNA were found to be hypersensitive to the aminoglycoside G418, but not to the non-aminoglycoside, chloramphenicol. Yeast expressing the *mar1-1* mutant cDNA were also hypersensitive, but this hypersensitivity was intermediate between wild-type *MAR1* yeast and empty vector controls.[23] Thus, the mar1-1 mutant protein, with single amino acid change, may still have partial functionality.

We developed a novel assay to detect antibiotic in chloroplast extracts, and used this assay to measure antibiotic content of chloroplasts from mutant, wild-type and *MAR1* overexpression lines. By spotting chloroplast lysates onto nitrocellulose membrane and using an antibody against gentamicin, we were able to show that lysates from mutant plants accumulated less gentamicin than wild-type, while overexpression lines hyper-accumulated gentamicin.[23] Taken together, our data illustrate that MAR1 does, in fact, act to transport aminoglycoside antibiotics into the chloroplast.

MAR1 Transports Antibiotic Opportunistically

It is, of course, unlikely that evolutionary pressures would have selected for a means of entry for toxic antibiotics into plant chloroplasts. Thus, is it probable that the transport of antibiotics via MAR1 is opportunistic in nature. We have found that *MAR1* overexpression lines are chlorotic, and this chlorosis can be rescued by iron supplementation. Additionally, *MAR1* expression is downregulated under limiting iron conditions.[23] We have therefore proposed that overexpression of *MAR1* effectively creates a condition of iron limitation in the chloroplast. Since iron deficiency is often associated with alterations in chloroplast ultrastructure, we have recently used TEM to investigate the chloroplast ultrastructure of MAR1 overexpression lines (35S::*MAR1*). When compared to wild-type, we observed highly disorganized and misaligned thylakoid membranes, lack of proper grana stacking, and an overall distended and distorted shape in 35S::*MAR1* chloroplasts (**Fig. 1**), all of which are symptoms of iron deficiency.[24-26]

We have previously proposed that the iron limitation in *MAR1* overexpressors could occur due to overaccumulation of an iron chelation molecule that aminoglycoside antibiotics are able to mimic structurally, thus obtaining entry into the

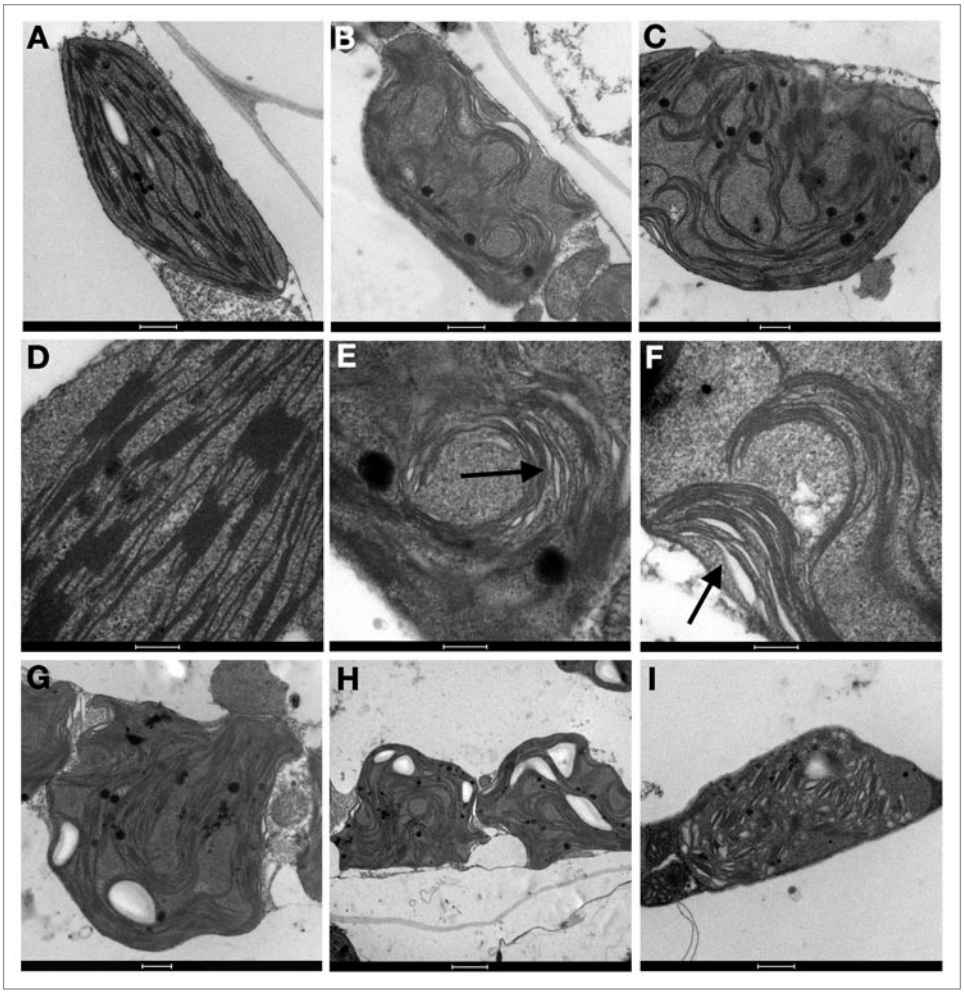

Figure 1. Chloroplast shape and ultrastructure in MAR1 overexpression lines are distorted to varying degrees. TEM images of chloroplasts from Ler wild-type (A and D) and 35S::*MAR1* (B, C and E–I). (E and F) are closeup images of (B and C), respectively. In (E and F), black arrows point to swollen lamellae. (I) illustrates swollen lamellae throughout, with no evidence of proper grana stacking. (G and H) illustrate gross shape distortion. Scale bars: (A–C), 500 nm; (D–F), 200 nm; (G and I), 500 nm; (H), 1 μm.

chloroplast. Because aminoglycosides are known to mimic polyamines, and may exploit polyamine inward transport systems to gain entrance to cells,[16] we have proposed that the elusive natural substrate of MAR1 may be the polyamine iron chelator, nicotianamine (NA). Mature *MAR1* overexpression lines have a leaf chlorosis pattern that is opposite that of the NA-less chloronerva mutant of tomato (*Lycopersicon esculentum*)[27]—instead of interveinal chlorosis in young tissues, chlorosis arises in the midvein and in older tissues.[23] We have hypothesized that this may be the result of a re-distribution of the cytoplasmic NA pool to the chloroplast, thus restricting NA from performing its role in phloem transport of iron and other metals.[28]

One of the *MAR1* homologs in Arabidopsis, *AtIREG1*, was postulated to be involved in vessel loading of iron,[29] and its downregulation in *DwMYB2* overexpressors may be the cause of the disruption in iron translocation (from root to shoot) observed in these plants.[30] Because iron is typically complexed with citrate in the xylem,[31] we feel it is possible that AtIREG1 exports citrate (or an iron-citrate conjugate) from root cells into the vasculature. Thus, AtIREG1 may be playing a role similar to FRD3, which mediates citrate efflux into root vasculature,[32] and is also downregulated in *DwMYB2* overexpressors.[30] More research is needed on AtIREG1 in order to explore this possibility. We postulate that the IREG proteins (AtIREG1, 2 and MAR1) may emerge as a family of transporters, distinct from the MATE and YSL families, which are also capable of transporting metal chelates or metal-chelate complexes.

Conclusions

Our work has begun to shed light on the possible mechanisms by which antibiotics—in this case, the particularly hydrophilic aminoglycosides—can cross membranes and reach their targets within plant cells. The MAR1 transporter is not solely dedicated to the transport of antibiotics, but rather, antibiotics are able to mimic the natural substrate of MAR1 to obtain entry into the chloroplast. It is interesting to note that many known drug and antibiotic efflux proteins in bacteria

also have a more "conventional" function. For example, the *E. coli* cmr chloramphenicol efflux pump bears sequence similarity to several sugar transporters.[33] The *B. subtilis* Blt drug transporter exports chloramphenicol and puromycin[34] as well as polyamines.[35] The *mexAB*/*oprM* multidrug efflux operon of *P. aeruginosa* is involved in efflux of tetracycline, chloramphenicol, several quinolones, and a range of β-lactams,[36] but is also regulated by iron concentration, and proposed to be involved in the secretion of the iron chelator, pyoverdine, under conditions of iron starvation.[37,38] Therefore, like these antibiotic export proteins, antibiotic importers may also be metabolite transporters that have been hijacked by antibiotic.

Our research on *MAR1* has contributed to knowledge regarding the processes of antibiotic entry in plants, which is currently in its infancy. Further knowledge about antibiotic entry pathways could eventually enable the production of crop plants that are incapable of antibiotic accumulation, thus protecting consumers and keeping our food supply safe and healthy. This knowledge may also aid us in development of new plant-based molecular markers, and generally contributes to the understanding of how plants interact with the antibiotics they encounter, both in the laboratory and in the natural environment.

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